Supplemental Information

Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice

Sarah E. Ross, Alan R. Mardinly, Alejandra E. McCord, Jonathan Zurawski, Sonia Cohen, Cynthia Jung, Linda Hu, Stephanie I. Mok, Anar Shah, Erin Savner, Christos Tolias, Roman Corfas, Suzhen Chen, Perrine Inquimbert, Yi Xu, Roderick R. McInnes, Frank L. Rice, Gabriel Corfas, Qiufu Ma, Clifford J. Woolf and Michael E. Greenberg.

Supplemental Data

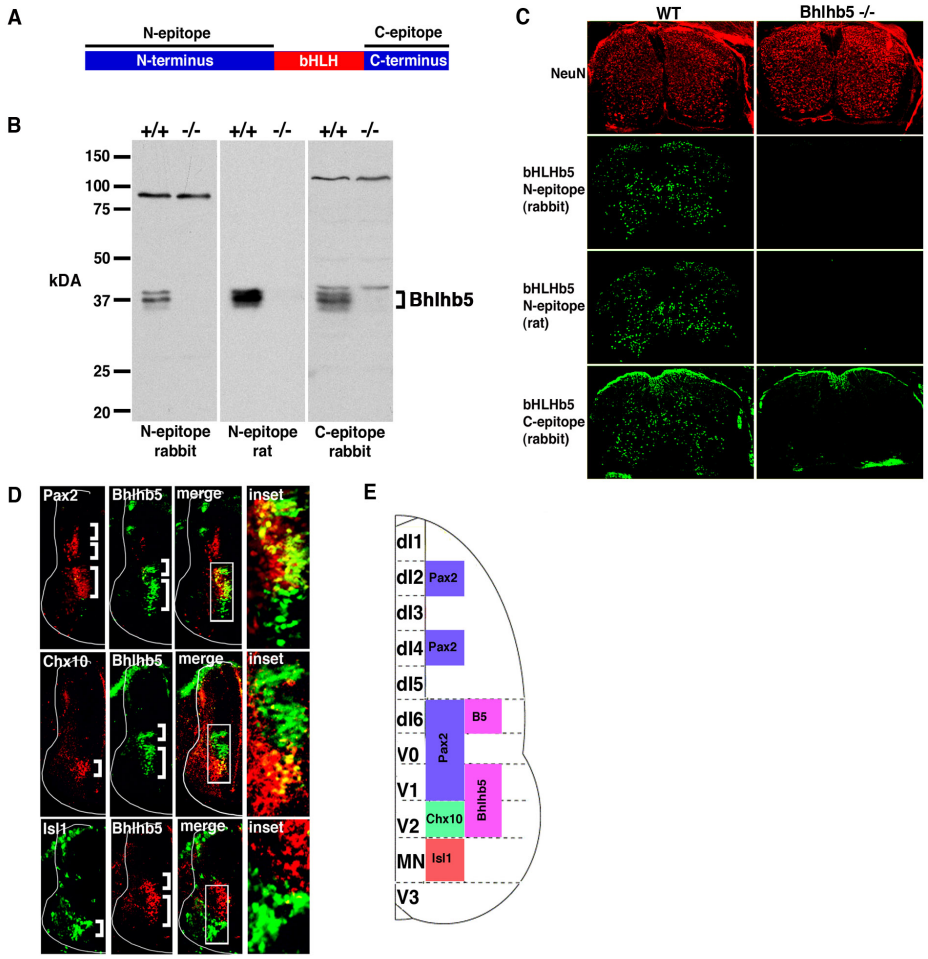

Figure S1. Generation of Bhlhb5-directed antibodies reveals that Bhlhb5 is expressed in V1, V2 and dI6 interneurons in the developing spinal cord.

Figure S1 related to Figure 1. A) Schematic of Bhlhb5 protein illustrating the basic helix-loop-helix domain (bHLH; red) and the N- and C-termini (blue). GST-fusion proteins to the N-terminal and C-terminal domains were used to generate Bhlhb5-directed antibodies. **B)** Western blot showing the specificity of Bhlhb5 antibodies. Cortical lysates from P0 wild type (+/+) or Bhlhb5 null (-/-) mice were subjected to immunoblotting using sera from rabbit injected with the N-epitope (left panel), rat injected with the N-epitope (middle), or rabbit injected with the C-epitope (right). Bhlhb5 runs as a triple on a 10% acrylamide gel, as indicated. **C)** Immunohistochemistry showing the specificity of Bhlhb5-directed antibodies. Lumbar spinal cord sections from P0 wild type (WT) or Bhlhb5 null (-/-) mice were stained with NeuN (top panel, red) or the indicated Bhlhb5 antibodies (green). **D)** Expression of Bhlhb5 in relation to various markers at E10.5. Spinal cord sections were stained with the indicated antibodies. Specific neuronal populations are denoted with white brackets and illustrated in the schematic in (E). Insets (boxed) are enlarged on the right. **E)** Summary of the expression of Bhlhb5 in relation to various markers.

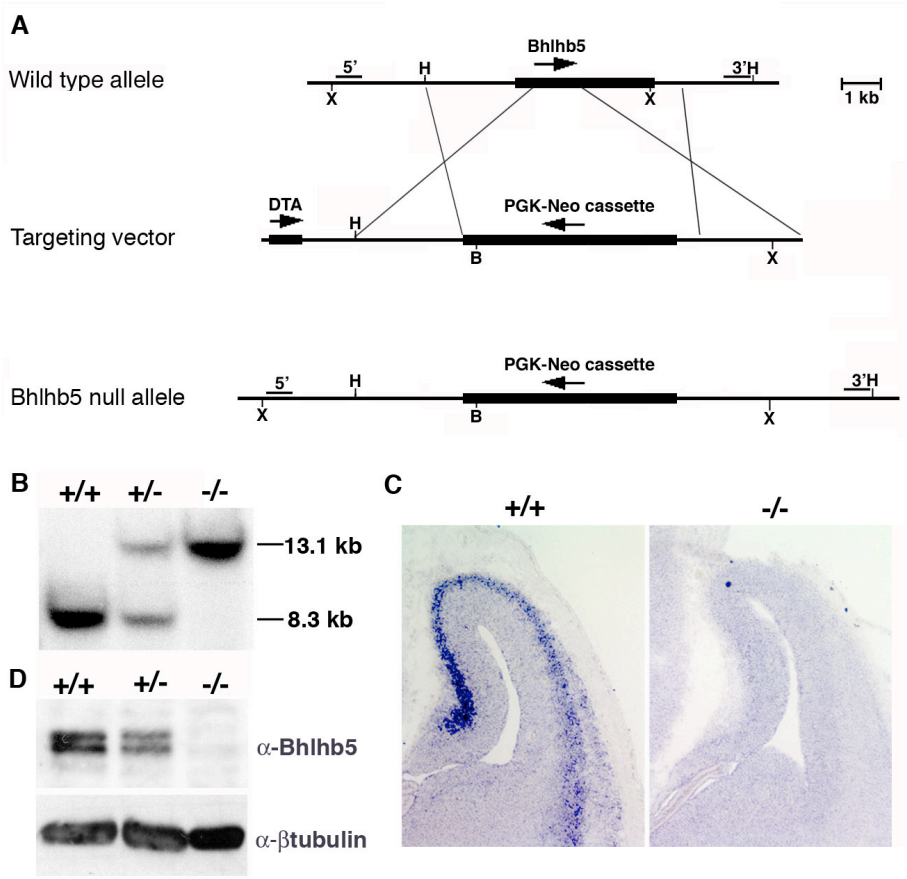

Figure S2. Generation of a Bhlhb5 null mouse

Figure S2 related to Figure 2. A) Targeting strategy for the introduction of a Bhlhb5 null allele into the Bhlhb5 locus by homologous recombination in ES cells. The Bhlhb5 gene, which is one exon, is denoted with a black rectangle. Open reading frames are indicated with arrows. H, HindIII restriction site; B, Bsu361 restriction site; X, Xba restriction site; 5', 5' Southern probe; 3', 3' Southern probe; PGK-Neo cassette, PGK promoter-neomycin positive selection cassette (note that this cassette also contains a βgalactosidase gene, which was not expressed for unknown reasons); DTA, diphtheria toxin negative selection cassette. **B)** Southern blot analysis of HindIII-digested genomic DNA from mice that are wild type (+/+), heterozygous (+/-) or Bhlhb5 null (-/-) using the 3' probe results in the predicted structures, indicating correct targeting. **C**) In situ hybridization for Bhlhb5 in coronal sections of the cortex from E13.5 wild type (+/+) or Bhlhb5 null (-/-) mice shows the loss of Bhlhb5 message in the cortical plate of Bhlhb5 mutants. **D)** Western blot analysis of cortical lysates from P0 mice that are wild type (+/+), heterozygous (+/-) or Bhlhb5 null (-/-) using Bhlhb5-directed antibodies or βtubulin antibodies, as a loading control. Bhlhb5 runs parallel to a 37 kDa marker and βtubulin runs just above a 50 kDA marker (not shown).

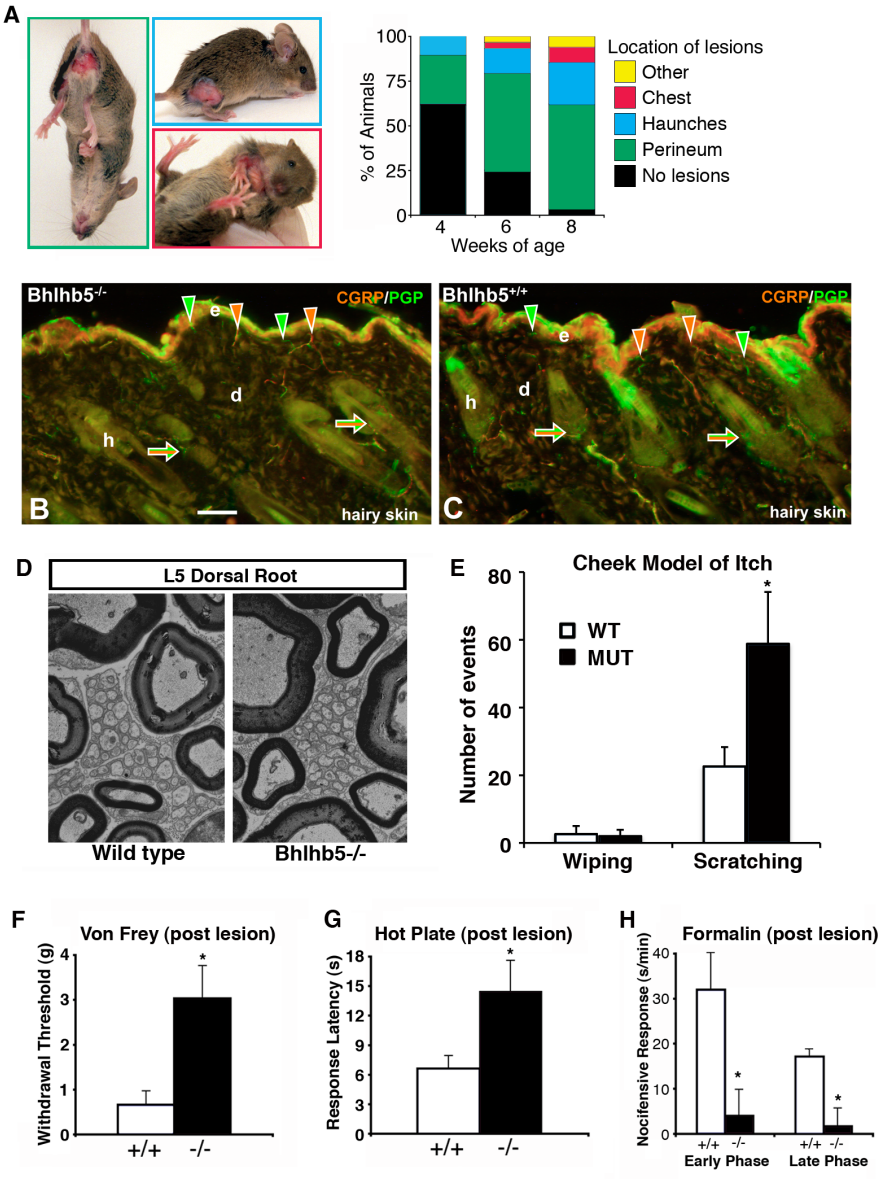

Figure S3. Initial characterization of Bhlhb5 -/- mice.

Figure S3 related to Figure 4. A) Map of the lesion sites in Bhlhb5 -/- mice. Incidence and location of lesions in Bhlhb5 mutant mice was analyzed at 4, 6 and 8 weeks. Note that all Bhlhb5 mutants eventually develop skin lesions. Although there are certain regions in which skin lesions are found most commonly (perineum, haunches and chest, as shown in photos), these lesions have been observed on virtually all body regions of Bhlhb5 -/- mice, including the orafacial area. **B - C)** Loss of Bhlhb5 has no obvious impact on cutaneous innervation in hairy skin. Immunofluorescent Images of hairy skin from the groin of Bhlhb5$^{-/-}$ and Bhlhl5$^{+/+}$ mice double labeled with antibodies for CGRP (red) and PGP (green). Thin caliber C fibers and Aδ fibers that label for CGRP and PGP (orange arrowheads) and only for PGP (green arrowheads) are present in the upper dermis of both genotypes where they innervate small blood vessel, the epidermis and the necks of hair follicles. Hair follicles in both phenotypes are innervated by piloneural complexes (orange and green stripped arrow) that are innervated by Aβ fibers that supply lanceolate endings and by CGRP positive and negative C fibers that supply circumferential endings. e, epidermis; d, dermis, h, hair follicle. Bar = 50μm. **D)** Electron micrographs of L5 dorsal roots from wild type and Bhlhb5 -/- mice at 6 weeks show no evidence of neuropathy. Quantification reveals that the number of myelinated and unmyelinated axons in L5 dorsal roots is not affected by the loss of Bhlhb5 (data not shown). Axons in the dorsal root, as visualized in sections from electron micrographs, were counted as a proxy for DRG neuron number. Dorsal roots from 4 pairs of mice were counted. No difference in the number of either myelinated or unmyelinated axons between Bhlhb5 -/- animals and their wild type littermates. **E)** Intradermal injection of 2.5% formalin (15 μl) into the cheek elicits predominantly a scratching response rather than a wiping response over the subsequent hour, suggesting that intradermal formalin may elicit an itch-like sensation. Bhlhb5 mutant mice scratch significantly more than wild-type mice ($p < 0.05$, t-test). Assay is based on Shimada and LaMotte (2008) . **F-H)** While young (4-week old) Bhlhb5 mutant mice show no significant defects in most nociceptive assays (Fig. 3), older (6 – 8 week) mutant mice that have skin lesions respond abnormally in assays for mechanical sensitivity and pain. Prior to our realization that the skin lesions in Bhlhb5 -/- mice are due to heightened itch, we performed a number of somatosensory assays using adult mice (6 – 8 weeks old), after the onset of skin lesions in Bhlhb5 -/- mice. These studies revealed that Bhlhb5 null (-/-) mice had significant defects in their responses in all tests compared to wild type (+/+) littermates, including mechanical sensitivity **(F)**, thermal sensitivity **(G)** and intraplantar formalin response **(H)**. Data are presented as mean ± SEM and * indicates a significant difference relative to controls ($p < 0.05$, t-test).

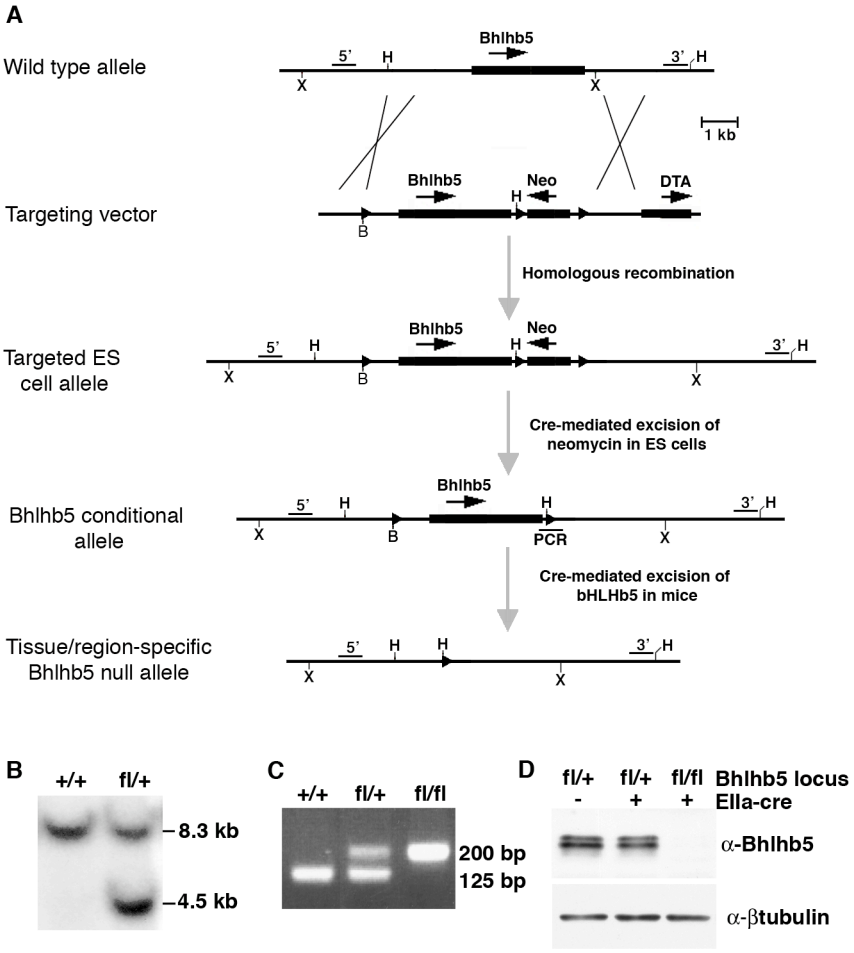

Figure S4. Generation of a conditional Bhlhb5 knockout

Figure S4 related to Figure 5. A) Targeting strategy for the introduction of a loxP-floxed Bhlhb5 conditional allele into the Bhlhb5 locus by homologous recombination in ES cells. The Bhlhb5 gene, which is one exon, is denoted with a black rectangle. LoxP sites are indicated with black triangles. Open reading frames are indicated with arrows. H, HindIII restriction site; B, Bsu361 restriction site; X, XbaI restriction site; 5', 5' Southern probe; 3', 3' Southern probe; Neo, neomycin positive selection cassette; DTA, diphtheria toxin negative selection cassette; PCR, region over which genotyping PCR is performed. **B)** Southern blot analysis of HindIII-digested genomic DNA from ES cells that are wild-type (+/+) or heterozygous floxed (+/fl) using the 3' probe results in the predicted structures, indicating correct targeting. **C**) PCR genotyping of genomic DNA isolated from tails of mice that are wild type (+/+), heterozygous floxed (fl/+), or homozygous floxed (fl/fl) shows a larger PCR product from the mutant allele than the wild-type allele due to additional bases associated with the remaining loxP site. **D)** EIIa-cre-mediated loss of Bhlhb5 gene results in the absence of Bhlhb5 protein. Western blot analysis of cortical lysates from P0 mice in which Bhlhb5 is either heterozygous floxed (fl/+) or homozygous floxed (fl/fl) and that also have (+) or lack (-) the EIIa-cre allele, as indicated, using Bhlhb5-directed antibodies or βtubulin antibodies, as a loading control. Bhlhb5 runs parallel to a 37 kDa marker and βtubulin runs just above a 50 kDA marker (not shown).

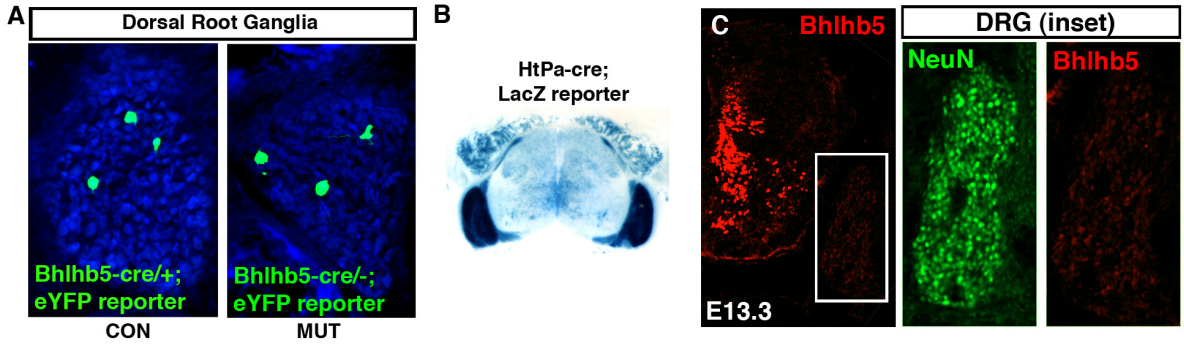

Figure S5. HtPa-cre causes recombination in DRG neurons, but not the spinal cord.

Figure S5 related to Figure 5. A) Bhlhb5-cre knockin reveals fate of Bhlhb5-cre marked cells in the dorsal root ganglia (DRG) of control (CON) and mutant (MUT) animals. A representative DRG from each genotype is stained for eYFP and counterstained with Hoechst. No difference in the number of cre-marked DRG neurons was observed in Bhlhb5 mutant mice. **B)** HtPa-cre-dependent expression of the LacZ reporter in P0 mouse reveals cre recombination throughout DRG neurons, but not in the spinal cord, confirming that the HtPA-mediated cre excision was specific. **C)** Upon HtPa-cre mediated recombination, Bhlhb5 is not expressed in DRG neurons, though it is expressed in the spinal cord. Spinal cord hemisection from E13.5 stained with Bhlhb5 (red) and NeuN (green). Inset, enlarged on right, reveals an absence of Bhlhb5 protein in DRG neurons.

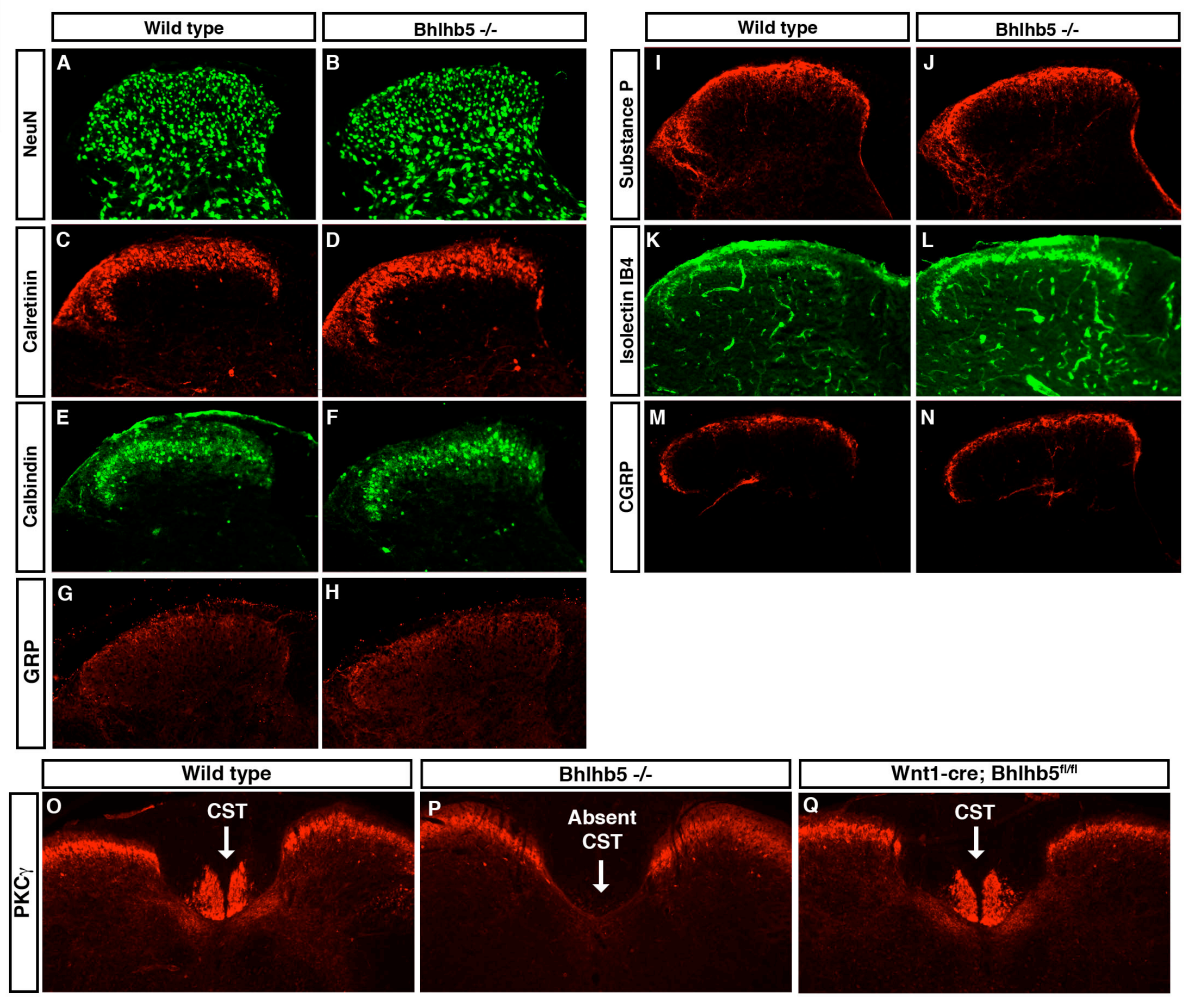

Figure S6. Bhlhb5 mutants show no dramatic defects in the dorsal horn but lack the corticospinal tract.

Figure S6 related to Figure 5. Immunostaining of wild type and Bhlhb5 mutant adult mice reveals that loss of Bhlhb5 has no obvious effect on the development of the dorsal horn. Representative lumbar sections are shown. **A, B)** Antibody staining for NeuN, a pan-neuronal marker. Antibody staining for the calcium binding proteins calretinin **(C, D)** and calbindin **(E, F),** which mark subsets of inhibitory neurons predominantly in lamina II. Antibody staining for GRP **(G, H)**, Substance P **(I, J)** and CGRP **(M, N)**, which are peptides expressed by primary afferents that terminate predominantly in lamina I and lamina IIo. Staining using fluorophore-conjugated isolectin IB4 (**K, L)**, which labels a subset of small sensory neuron that that terminate predominantly in lamina IIi. Antibody staining for PKCγ **(O, P, Q),** which is expressed in a subset of neurons in the dorsal horn. In addition, PKCγ is expressed in the axons of neurons that form the corticospinal tract (CST), which extends down the spinal cord in the dorsal funiculus (arrows). Note that the CST appears to be missing in mice that constitutively lack Bhlhb5, but not upon Wnt1cre-mediated loss of Bhlhb5, consistent with the possibility that the CST defect is due to a cell autonomous lack of Bhlhb5 in corticospinal motor neurons. The absence of a CST in Bhlhb5 mutant mice was confirmed using the Bhlhb5-cre mouse and Rosa-eYFP reporters, which revealed that in Bhlhb5 mutants the corticospinal axons terminate prematurely, extending only as far as the pyramidal decussation (data not shown). The finding that Bhlhb5 mutants are missing the corticospinal tract was also recently reported by (Joshi et al., 2008).

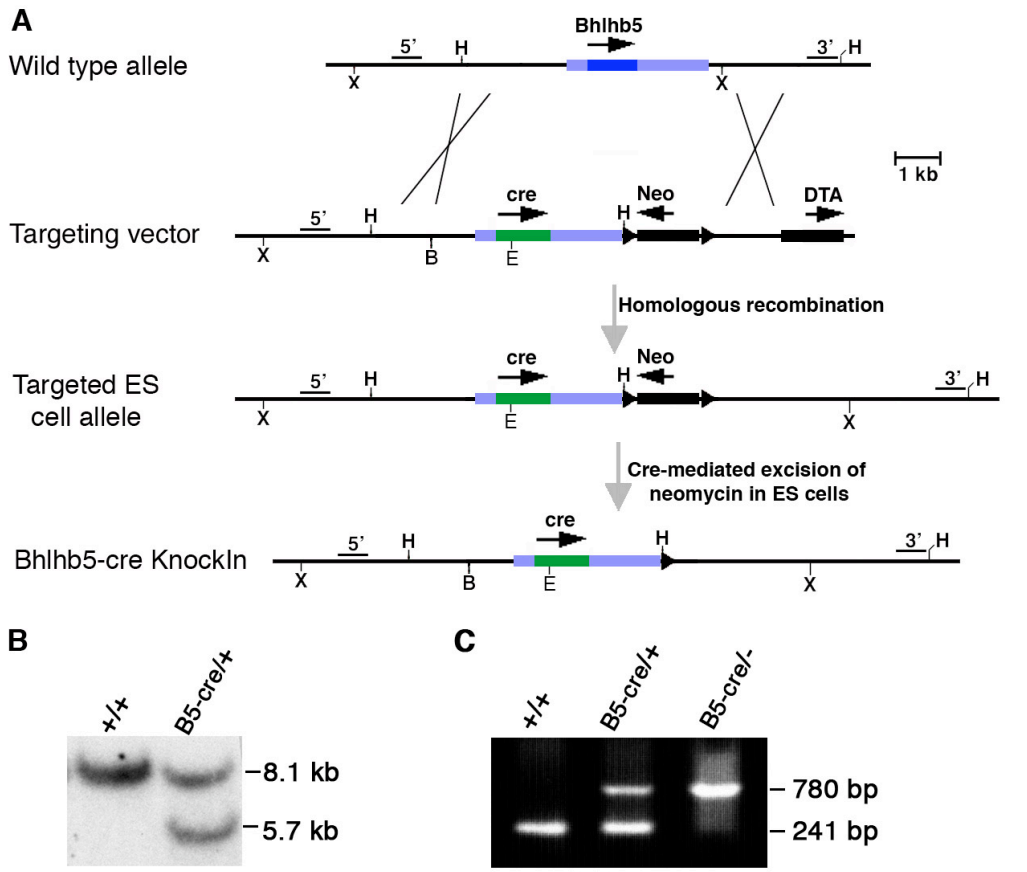

Figure S7. Generation of a Bhlhb5-cre knockin

Figure S7 related to Figure 6. A) Targeting strategy to replace the Bhlhb5 open reading frame with that of cre recombinase. Open reading frames are indicated with arrows and dark shaded regions within rectangles for Bhlhb5 (blue) and cre (green). LoxP sites are indicated with black triangles. H, HindIII restriction site; E, EcoRV restriction site; X, XbaI restriction site; 5', 5' Southern probe; 3', 3' Southern probe; Neo, neomycin positive selection cassette; DTA, diphtheria toxin negative selection cassette. **B)** Southern blot analysis of EcoRV- and XbaI-digested genomic DNA from ES cells that are wild-type (+/+) or heterozygous for the Bhlhb5-cre allele (B5-cre/+) using the 5' probe results in the predicted structures, indicating correct targeting. **C**) PCR genotyping of genomic DNA isolated from tails of mice that are wild type (+/+), heterozygous for the Bhlhb5-cre allele (B5-cre/+), or mutants harboring one copy of the Bhlhb5-cre allele and one copy of the Bhlhb5 null allele (B5-cre/-) shows PCR products of the expected size. This PCR amplifies the junction between the 3' UTR and the cording region of either the Bhlhb5 gene of the cre recombinase gene.

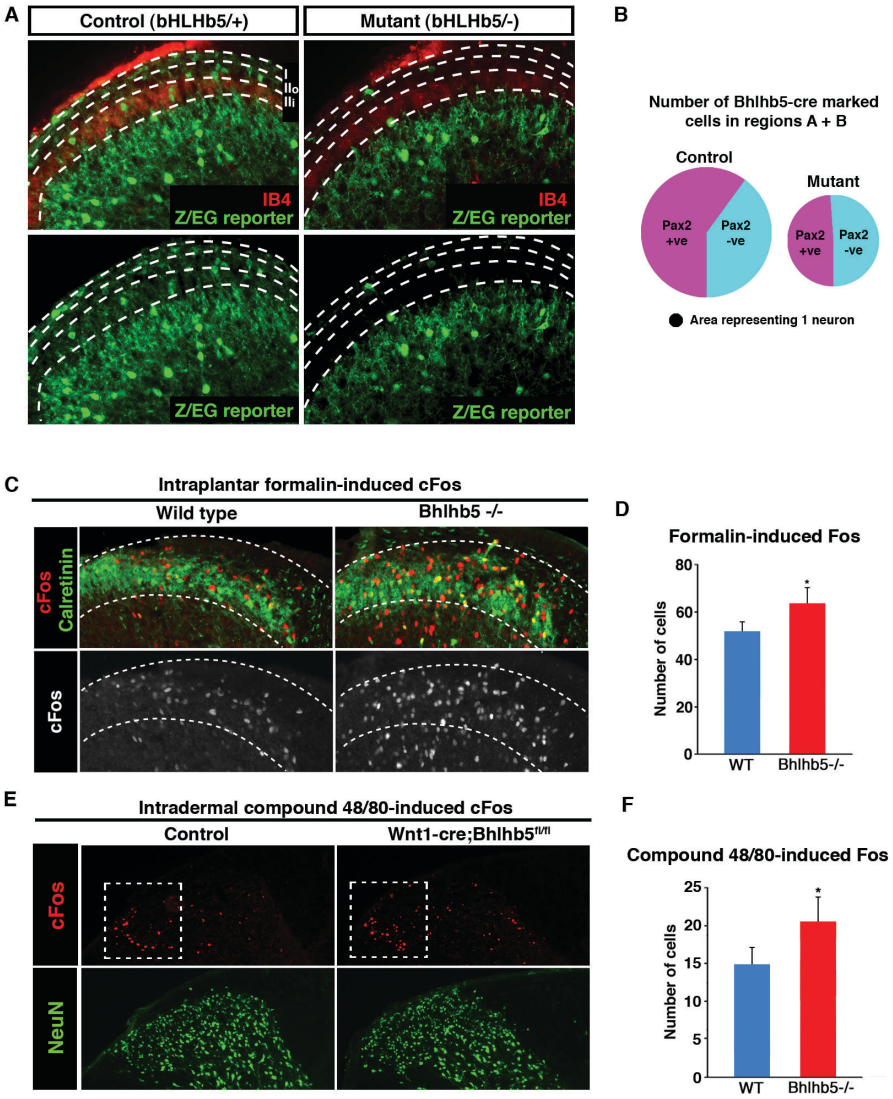

Figure S8. Insight into the role of Bhlhb5-expressing neurons of the dorsal horn

Figure S8 related to Figure 6. A) Z/EG reporter reveals a loss of Bhlhb5cre-expressing neurons in laminae I and II. Laminar distribution of Bhlhb5-cre marked cells in the dorsal horn of control and mutant mice at P28 visualized with the cre-responsive Z/EG reporter. Isolectin-IB4 (red; lamina IIi) and CGRP (not shown; lamina I and IIo) were used as markers to subdivide the spinal cord into laminae, denoted by the doted lines. In the Bhlhb5 mutant, cell bodies and neuropil are missing in lamina I and II. **B)** Both excitatory and inhibitory neurons are lost from the dorsal horn of Bhlhb5 mutant mice. Quantification of Bhlhb5-cre-marked cells that either co-label with Pax2 (Pax2 +ve; inhibitory neurons) or do not co-label with Pax2 (Pax2 –ve; mostly excitatory neurons) in control and Bhlhb5 mutant mice is shown. The area of the pie graph represents the number of neurons. Note that the number of Bhlhb5-cre-marked neurons (area of the pie chart) is reduced in the Bhlhb5 mutants, but there is no dramatic effect on the relative proportion of Pax2-expressing neurons, indicating that both excitatory and inhibitory neurons are missing from the dorsal horn of Bhlhb5 mutant mice. **C, E)** Elevated formalin- and compound 48/80-induced c-Fos expression in the dorsal horn of Bhlhb5 -/- mice. To help determine whether the heightened behavioral response observed upon either the intraplantar injection of formalin or the intradermal injection of compound 48/80 in Bhlhb5 -/- mice was due to defects within the circuitry of the dorsal horn, c-Fos-induction in the dorsal horn was analyzed as an indirect measure of neuronal activity. To quantify c-Fos induction in response to intraplantar formalin, matched lumbar sections were double labeled with antibodies to c-Fos and the calretinin (a lamina II marker). **D, F)** Quantification of c-Fos induction following the injection of formalin or compound 48/80. In Bhlhb5 mutant mice there was a significant increase in the number of c-Fos-expressing neurons in the superficial dorsal horn two hours following treatment. For formalin, c-Fos-expressing cells within or above a region defined by calretinin staining were quantified, whereas, for compound 48/40, c-Fos-expressing cells within a square region as illustrated by the dotted were quantified. Quantification was performed in a blinded fashion using Metamorph software and counting 8-10 matched sections/animal, taken every 200 microns. Data are presented as mean ± SEM and * indicates a significant difference relative to controls ($P < 0.05$, t-test).

Supplemental Table

Bhlhb5 alleles	Primers	Sequence	Product
Bhlhb5 null allele	Forward Wt-reverse Mut-reverse	5'-GGTGAATCCAAGCAAGATAAACGG-3' 5'-ATCAGCGGGCTCGAAACAGC-3' 5'-TTGGGTAACGCCAGGGTTTTCC-3'	Wt: 534 bp Mut: 338 bp
Bhlhb5 conditional allele	Forward Reverse	5'-ACTAAGAGGACAAGAACTGGG-3' 5'-TGTGGGAGAGACTTTCAGG-3'	Wt: 125 bp Mut: 200 bp
Bhlhb5-cre knockin allele	Forward Wt-reverse Mut-reverse	5'-CCTGACTCTCCAGCCCAGGTG-3' 5'-ATCAGCGGGCTCGAAACAGC-3 5'-GGCAACACCATTTTTTCTGACC-3'	Wt: 241 bp Mut: 280 bp

Table 1. Primers used for genotyping Bhlhb5 alleles

Supplemental Experimental Procedures

Generation of Bhlhb5-directed antibodies
GST-fusion proteins encompassing either amino acids 1-209 (for the N-terminal epitope) or 261-355 (for the C-terminal epitope) of Bhlhb5 were purified from bacteria and injected into rabbits or rats (Figure S1A). The specificity of these antibodies was confirmed using the Bhlhb5 knockout (Figures S1B and S1C).

Generation of Bhlhb5 null, Bhlhb5 conditional knockout, and Bhlhb5-cre knockin mice
The targeting vectors were constructed from 129s6/SvEvTAC mouse genomic fragments amplified by PCR. For the Bhlhb5 null construct, the entire Bhlhb5 gene was replaced with a βgalactosidase-neomycin cassette. Specifically, a 2,7 kb 5' genomic fragment (from HindIII to NotI) and a 2.6 kb 3' genomic fragment (XhoI to SalI) were introduced on either side of the selection cassette (Figure S2A). Note that, for unknown reasons, βgalactosidase protein was not expressed in mice harboring Bhlhb5 null allele. For the Bhlhb5 conditional knockout construct, a 41 base oligonucleotide containing a loxP site and a novel Bsu361 site was introduced between the PstI and BamHI sites, 1 kb upstream of the Bhlhb5 gene within a region that was unconserved. A loxP-flanked PGK-neomycin cassette was introduced into the XbaI site downstream of the 3' UTR in a region that was unconserved. An additional 1.8 kb genomic fragment (XbaI to EcoRV) was introduced 3' to the selection cassette. Finally, a diphtheria toxin negative selection cassette (PGK-DTA) was introduced 3' to the genomic fragment (Figure S4A). For the Bhlhb5-cre knockin construct, the coding region of the Bhlhb5 gene was replaced with the coding region of the cre recombinase gene by fusion PCR. In other respects, this targeting construct was identical to that of the conditional knockout except that it lacked the 5' loxP site (Figure S7A). More details are available upon request.

All targeting constructs were confirmed by direct sequencing of their entirety prior to use in gene targeting. Linearized targeting vectors were electroporated into sv129 mouse ES cells. For each of the three alleles, G418-resistant ES cell clones were screened for homologous recombination at the Bhlhb5 locus by Southern blotting using both 3' and 5' probes external to the genomic fragment contained within the targeting vector. Recombinant clones containing the predicted rearranged fragment were obtained at frequencies of around 10 %. Primers used to amplify the 5' probe were F: 5'- TGCCTGGTTCCTTCTTCTTCCG-3' and R: 5'- GCAGCCCCAAGTAATGTGTAGG-3'. Primers used to amplify the 3' probe were F: 5'- AGGCTCCATCTACCTTGTGAGTCC-3' and R: 5'-GGCTTCATTACCTCCCTCTTTGC-3'. For the Bhlhb5 null allele, the 5' arm was screened by cutting with XbaI and Bsu361; correct re-arrangement shifted the resulting genomic fragment from 8.1 kb to 5.5 kb; the 3' arm was screened by cutting with HindIII; correct re-arrangement shifted the resulting genomic fragment from 8.3 kb to 13.1 kb (Figure S2B). For the Bhlhb5 conditional knockout allele, the 5' arm was screened by cutting with XbaI and Bsu361; correct re-arrangement shifted the resulting genomic fragment from 8.1 kb to 3.4 kb; the 3' arm was screened by cutting with HindIII; correct re-arrangement shifted the resulting in a genomic fragment from 8.3 kb to 4.5 kb (Figure S4B). For the Bhlhb5-cre knockin construct, the 5' arm was screened by cutting with EcoRV and XbaI; correct re-arrangement shifted the resulting genomic fragment from 8.1 kb to 5.7 kb; the 3' arm was screened by cutting with HindIII; correct re-arrangement shifted the resulting genomic fragment from 8.3 to 4.5 kb (Figure S7).

For each mutant, two positive ES cell clones were independently transfected with a cre recombinase-expressing plasmid and appropriate excision of the loxP-flanked neomycin cassette was confirmed by PCR. Two confirmed ES cell clones per mutant were injected into C57BL/6 blastocysts to generate two independent lines per mutant. These ES cells were subsequently implanted into pseudopregnant females to generate chimeric offspring, which were screened for the mutation by PCR genotyping (see below).

Animal husbandry and colony management

For routine experimentation, animals were genotyped using a PCR-based strategy. Animals harboring the Bhlhb5 null allele were genotyped with a forward primer in the Bhlhb5 proximal promoter and a reverse primer in either the Bhlhb5 gene. Conditional knockout animals were genotyped for the presence of the loxP site, which shifts the size of the PCR product. Animals harboring the Bhlhb5-cre allele were genotyped with a forward primer in the Bhlhb5 proximal promoter and a reverse primer in either the Bhlhb5 gene or the cre recombinase gene. See Table 1 for primer sequences and PCR product sizes. Also see Figures S2C, S4C, and S7C. The use of animals was approved by the Animal Care and Use Committee of the Children's Hospital and/or that of Harvard Medical School.

Immunohistochemistry and Xgal Staining

For immunocytochemistry, mice were fixed with 4% paraformaldehyde in PBS by intracardial perfusion. For X-gal staining, mice were perfused with 1% PFA, 0.2% gluteraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in PBS. For adult animals, spinal cords were dissected. For embryonic animals, spinal cords were left in vertebra, but removed from other tissue. Tissues were post-fixed for 1 h at 4 degrees, washed extensively with PBS, cryopreserved in 20% sucrose in PBS overnight, embedded in OTC, and frozen. Sections were cut at 20 microns on a cryostat and placed on slides. For immunostaining, sections were blocked in 10% goat serum and 0.25% triton-X in PBS for 1 h at RT. Sections were incubated with primary antibodies in block overnight at 4° C. Slides were washed 4 times 5 min in PBS containing 0.1% triton-X. Detection was carried out using Alexa-fluor secondary antibodies diluted 1 : 500 in block. Sections were counterstained with the nuclear dye Hoechst 33342 for one minute at room temperature. Sections were washed, as above, and coverslipped. Primary antibodies were as follows: Bhlhb5 amino-terminal antibody (rat, 1:1000, or rabbit, 1:10,000 generated in the Greenberg lab); NeuN (mouse, 1:1000, Chemicon); c-Fos (rabbit, 1:1000 SantaCruz) calretinin (rabbit, 1:1000, Chemicon) 1:1000; calbindin (rabbit, 1:1000, Chemicon); CGRP (rabbit, 1:1000, Immunostar) 1:000; substanceP (rabbit, 1:1000, Immunostar); Prdm8 (1:500, rabbit, generated in the McInnes lab); isolectin-IB4 (Alexa-fluor conjugated, 1:200, Invitrogen); GFP (which also recognizes YFP, Chicken, 1:1000, Aves labs); cleaved caspase-3 (rabbit, 1:1000, Cell Signaling); Pax2 (rabbit, Zymed); Lmx1b (guineu pig, 1:16,000, Jessell lab). For X-gal staining, sections were washed three times in wash buffer (0.2% triton-X, 5 mM DTT, 1 mM $MgCl_2$ in PBS) and then incubated in wash buffer containing 1 mg/ml X-gal, 5 mM ferrocyanide and 5 mM ferricyanide at 37 ° C for several hours.

Quantification of neurons and c-Fos induction

For neuronal and c-Fos quantification experiments, 3 - 5 pairs of littermates were analyzed. Eight to ten matched sections per animal were used for quantification, spanning 1600-2000 microns. Cell counts in various regions of the spinal cord were conducted using the cell count function of Metamorph (Molecular Devices). Metamorph cell scoring parameters were validated by manual counts and were kept constant across all conditions. For c-Fos induction experiments, mice received 25 µl intraplantar injection of 5% formalin or 100 µg of compound 48/80 intradermally into the nape of the neck. Two hours later, mice were perfused and processed for immunohistochemistry, as described above. To quantify c-Fos induction in response to intraplantar formalin, matched lumbar sections were double labeled with antibodies to c-Fos and the calretinin (a lamina II marker) and quantified the number of c-Fos expressing neurons in or above the calretinin-defined zone. To quantify c-Fos induction in response to intradermal compound 48/80, c-Fos-expressing cells within a square region at the lateral edge of the dorsal horn were quantified, as illustrated in Figure S8E. Data are presented as mean ± SEM and * indicates a significant difference relative to controls ($P < 0.05$, t-test). All counts were conducted blind to genotype.

Behavioral Experiments

Where appropriate, mice were habituated for 20 min/day over several days. For the cheek model of itch, 5 pairs of 4 week-old mice were analyzed. Dilute formalin (2.5%, 15 µl) was injected intradermally into the cheek and monitored for the subsequent hour. Bouts of scratching mediated by the hindpaw were counted as scratching, whereas wiping events mediated by the forearm were counted as wiping, as described (Shimada and LaMotte, 2008). Several behavioral experiments (Von Frey, Hot Plate, Intraplantar Formalin Test) were performed on older mice (6 – 8 weeks old, subsequent to the onset of lesions in Bhlhb5 mutant mice. For these experiments, 6 – 8 pairs of littermates were analyzed. To assess mechanical sensitivity, calibrated von Frey fibers were applied to the plantar surface of the hindpaws of mice. The smallest monofilament that evoked paw-withdrawal responses on five out of ten trials was taken as the mechanical threshold. To determine thermal pain threshold, mice were placed on a 55 °C hot plate and

the response latency to paw licking or jumping was recorded. For the formalin test, the mice received 25 µl intraplantar injection of 5% formalin, and the licking behavior of the injected paw was recorded in five minute intervals for 1 hr for the subsequent hour.

Supplemental References

Joshi, P.S., Molyneaux, B.J., Feng, L., Xie, X., Macklis, J.D., and Gan, L. (2008). Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. Neuron *60*, 258-272.

Shimada, S.G., and LaMotte, R.H. (2008). Behavioral differentiation between itch and pain in mouse. Pain *139*, 681-687.